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Keywords:	adenosine deaminase, cognitive performance, plasticity, slow wave sleep, synaptic homeostasis

FUNCTIONAL *ADA* POLYMORPHISM INCREASES SLEEP DEPTH AND REDUCES VIGILANT ATTENTION IN HUMANS

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Abstract

Homeostatically regulated slow-wave oscillations in nonREM sleep may reflect synaptic changes across the sleep-wake continuum, and the restorative function of sleep. The non-synonymous c.22G>A polymorphism (rs73598374) of adenosine deaminase (ADA) reduces the conversion of adenosine to inosine, and predicts baseline differences in sleep slow-wave oscillations. We hypothesized that this polymorphism affects cognitive functions, and investigated whether it modulates EEG, behavioral, subjective, and biochemical responses to sleep deprivation. Attention, learning, memory, and executive functioning were quantified in healthy adults. Right-handed carriers of the variant allele (G/A genotype, n = 29) performed worse on the d2 attention task than G/G homozygotes (n = 191). To test whether this difference reflects elevated homeostatic sleep pressure, sleep and sleep EEG before and after sleep deprivation were studied in 2 prospectively matched groups of G/A and G/G genotype subjects. Deep sleep and EEG 0.75-1.5 Hz oscillations in nonREM sleep were significantly higher in G/A than in G/G genotype. Moreover, attention and vigor were reduced, whereas waking EEG alpha activity (8.5-12 Hz), sleepiness, fatigue, and α-amylase in saliva were enhanced. These convergent data demonstrate that genetic reduction of ADA activity elevates sleep pressure and plays a key role in sleep and waking quality in humans.

Keywords: adenosine deaminase; cognitive performance; plasticity; slow wave sleep; synaptic homeostasis

Introduction

Sleep homeostasis refers to the general principle that elevated sleep need following sleep loss is counteracted by prolonged sleep duration and, especially, by enhanced sleep intensity (Borbély 1980, 1982; Daan et al. 1984). A highly predictable and reliable marker of non-rapid-eye-movement (nonREM) sleep intensity is the prevalence of slow-wave oscillations in the electroencephalogram (EEG). Accumulating evidence indicates that EEG slow-wave oscillations in nonREM sleep are causally linked to local synaptic processes, which reflect the duration and quality of prior wakefulness (Rao et al. 2007; Massimini et al. 2009). Homer 1a, brain-derived neurotrophic factor (BDNF), and other molecular markers of brain plasticity were recently suggested to play causal roles in sleep homeostasis (Maret et al. 2007; Faraguna et al. 2008).

Slow (delta) wave oscillations characterizing deep nonREM sleep consist at the cellular level of rhythmic alternations in the membrane potential of cortical neurons between a hyperpolarized down-state and a depolarized up-state (Steriade et al. 1993). Given their tight homeostatic regulation (Bersagliere and Achermann 2010), slow waves are thought to be essential for the functions of sleep, which may include synaptic homeostasis, learning, and consolidation of memories (Tononi and Cirelli 2006; Diekelmann and Born 2010). In support of these views, pharmacological and electrical induction of slow-wave oscillations during sleep may potentiate memories and reduce the detrimental consequences of sleep restriction on cognitive performance (Marshall et al. 2006; Walsh et al. 2006; Walsh et al. 2010). By contrast, sleep deprivation and experimental slow wave suppression may impair many cognitive abilities such as attention, perceptual processing and learning, hippocampal activation, and memory encoding (Aeschbach et al. 2008; Landsness et al. 2009; Tomasi et al. 2009; Van Der Werf et al. 2009).

The molecular and neurochemical bases of sleep homeostasis, and the relationships between waking brain activity and sleep slow-wave oscillations are poorly understood. However, adenosine and its receptors play a well-established role in sleep homeostasis (Basheer et al. 2004; Landolt 2008). Moreover, *in vitro* data show that for example the facilitatory action of BDNF on long-term potentiation (LTP) requires endogenous adenosine. More specifically, enhanced LTP by BDNF in

hippocampal slices was prevented when adenosine was removed with the adenosine metabolizing enzyme adenosine deaminase (ADA) (Fontinha et al. 2008). Thus, it is possible that BDNF and the adenosine neuromodulator system interact to mediate the consequences of neural activity during wakefulness on the homeostatic regulation of nonREM sleep.

Adenosine kinase (ADK) and ADA contribute to the regulation of extracellular adenosine levels (Fredholm et al. 2005). Not only ADK (Palchykova et al. 2010) but also ADA may be involved in homeostatic sleep-wake regulation. Converging genetic and pharmacological studies in mice and rats indicate an important role for Ada in regulating the build-up of nonREM sleep need during prolonged wakefulness, as well as nonREM sleep intensity (Franken et al. 2001; Okada et al. 2003). In humans, a functional G>A transition at nucleotide position 22 of the coding sequence of the *ADA* gene (c.22G>A; rs73598374) is associated with enhanced slow-wave activity in baseline sleep (Rétey et al. 2005). While these data suggest that this genetic variation increases nonREM sleep pressure, the answers to the questions whether it also affects cognitive performance and sleep homeostasis are unknown.

Heterozygous G/A allele carriers of *ADA* show reduced ADA enzymatic activity (Battistuzzi et al. 1981; Riksen et al. 2008) and may have higher endogenous adenosine levels than homozygous G/G genotype subjects (Hirschhorn et al. 1994). We investigated the functional consequences of the c.22G>A polymorphism in 220 healthy volunteers, and systematically recorded self-reported sleep-wake habits and quantified cognitive abilities including attention, learning, memory, and executive functioning. In a subsequent case-control study in 22 healthy adults, 4-week-rest-activity patterns and homeostatic sleep-wake regulation were studied by quantifying neurophysiological, behavioral, subjective, and biochemical responses to a night without sleep. Based on the previous findings, we hypothesized that the G/A genotype subjects would exhibit higher sleep pressure and habitually sleep longer, show more slow wave sleep and slow-wave activity, and be more strongly affected by sleep deprivation than the G/G homozygotes.

Materials and Methods

Subject recruitment and genotyping

The Cantonal ethics committee for research on human subjects reviewed and approved the study protocol and all experimental procedures. They were conducted according to the principles of the Declaration of Helsinki. All participants provided written informed consent.

One-hundred-twenty-seven men and 118 women were genotyped. The prevalence of the G/A genotype was roughly 13 % (31/245), whereas 87 % had the G/G genotype (214/245) and no individual with A/A genotype was present. These genotype frequencies are in accordance with previous findings in a healthy Italian population (Persico et al. 2000). Self-reported sleep-wake habits, attention, learning, memory, and executive performance were systematically quantified. Because we were also interested in lateralized cognitive functions (see supporting information, Table S1) and right-handedness guarantees a uniform degree of functional lateralization, only the data of right-handed individuals (n=220) were analyzed. Among the G/A genotype subjects, 5 healthy women and 6 healthy men (all Swiss or German) willing to participate in a sleep deprivation study were recruited for the laboratory experiment. They were prospectively matched with 11 G/G homozygotes with regard to sex, age, years of education, habitual alcohol and caffeine intake, body-mass index, trait anxiety, diurnal preference and daytime sleepiness (see supporting information, Table S2). Women were matched with respect to the phase in the menstrual cycle (follicular phase, luteal phase). All participants reported to have no history of neurological and psychiatric disease, not taking any medication, and to be moderate alcohol and caffeine consumers. Two pairs of subjects were matched with respect to cigarette smoking (~ 10 cigarettes per day); all other participants were non-smokers.

Genomic DNA was extracted from 3 ml fresh EDTA blood with the Wizard® Genomic DNA purifcation kit (Promega, Madison, WI, USA). Genotypes were determined by allele-specific polymerase chain reaction on a MJ Research PTC-225 thermal cycler (MJ Research / Bio-Rad, Reno, NV, USA). HOT FIREPol® DNA polymerase and the following primers were used: forward primer, 5'-gcgcacgagggcaccat-3'; reverse primer, 5'-gaactcgcctgsaggagcc-3' (annealing temperature, 67° C). Sequencing was performed by the Sanger chain-termination method (Sanger et al. 1977) with an ABI

PRISM® 3100 (16 capillaries) (Applied Biosystems Inc., Foster City, CA, USA) genetic analyzer. All genetic analyses were replicated at least once for independent confirmation of the results.

Habitual sleep duration

Self-reported habitual sleep length on work and leisure days was quantified with the Munich Chronotype Questionnaire (Roenneberg et al. 2003). All participants of the sleep deprivation study wore during 4 weeks a rest-activity monitor (Actiwatch, Cambridge Neurotechnology Ltd, Cambridge, United Kingdom) on the wrist of their non-dominant arm. Habitual sleep length was estimated form the records of the rest-activity patterns, together with inspection of sleep-wake diaries. Note that actigraphy-derived "sleep duration" refers to total time in bed, including possible brief intrusions of wakefulness.

Attention, learning, memory, and executive functioning

The cognitive abilities of all study participants were systematically tested during 2 hours.

The d2 attention task (Brickenkamp 1962) is a timed test of selective attention. Fourteen lines of the letters "d" and "p" with 1-4 dashes arranged either individually or in pairs above and below the letters are presented on a sheet of paper. The subjects are allowed 20 s to scan each line, and to mark all letters "d" with 2 dashes. Outcome measures include the total number of processed items, a highly reliable measure of processing speed, the sums of errors of omission and commission, as well as the fluctuation rate across trials (Brickenkamp 1962).

The validated tests used to assess verbal and non-verbal learning efficiency, working memory and executive functions included the Rey Auditory Verbal Learning Test (Strauss et al. 2006) the Rey Verbal Design Learning Test (Foster et al. 2009), the Digit Span Test (Strauss et al. 2006), a Stroop Color-Word Task (Stroop 1935; Perret 1974), the Random Number Generation task (Towse 1998), a Go/No-Go Test (Greenwald et al. 1998), a Design Fluency Test (Regard et al. 1982), and a Letter Fluency Test (Perret 1974).

Laboratory study to examine homeostatic sleep-wake regulation

All participants of the laboratory study were polysomnographically screened in the sleep lab, to exclude poor sleep efficiency and unrecognized, pre-existing sleep disorders.

The experimental protocol consisted of 4 nights and 2 days in the sleep laboratory. The first and second nights (24:00-08:00) served as adaptation and baseline, respectively. The subsequent 2 days and 1 night, subjects were not allowed to sleep. During the 40 hours prolonged wakefulness, they were constantly supervised by members of the research team. Subjects were free to read, study, play games, watch movies and occasionally take a walk outside the laboratory. They received normal meals 3 times a day, prepared either in the University cafeteria or by themselves in a kitchen adjacent to the laboratory. The last night (24:00-10:00) served as recovery night from sleep deprivation.

Pre-study procedures

For two weeks prior to the study, volunteers were asked to abstain from all sources of caffeine (coffee, tea, cola drinks, chocolate, and energy drinks), to wear a wrist activity monitor on the non-dominant arm, and to keep a sleep-wake diary. For 3 days before and during the study, all subjects had to also abstain from alcohol, and to maintain regular 8:16-hours sleep-wake cycles. Bed times were scheduled from 24:00-08:00. When not adhering to the directives, subjects were excluded from the study. The smokers were asked to write down the number of cigarettes per day (not more than ~ 10 cigarettes per day were allowed). During the study, the two pairs of smokers were allowed to smoke at the same pre-defined times, in order to avoid withdrawal.

All-night polysomnography

Polysomnographic recordings including EEG, bipolar electrooculogram (EOG), mental electromyogram (EMG) and electrocardiogram (ECG) were continuously conducted during all

experimental nights, with Rembrandt Datalab® (Version 8; Embla Systems, Broomfield, CO, USA) and the polygraphic amplifier Artisan® (Micromed, Mogliano Veneto, Italy). Analog signals were conditioned by a high-pass filter (EEG: -3 dB at 0.15 Hz; EMG: 10 Hz; ECG: 1 Hz) and an anti-aliasing low-pass filter (-3 dB at 67.2 Hz), digitized and transmitted via fiber-optic cables to a personal computer. Data were sampled with a frequency of 256 Hz. The EEG was recorded from 1 referential (C3A2) and 8 bipolar derivations along the left and right anteror-posterior axes. The data derived from the C3A2 derivation are reported here.

Sleep stages (Rechtschaffen and Kales 1968) were visually scored for 20-s epochs with Rembrandt Analysis Manager® (Version 8; Embla Systems, Broomfield, CO, USA). Four-s EEG spectra (FFT routine, Hanning window, 0.25-Hz resolution) were calculated with MATLAB® (The MathWorks Inc, Natick, MA, USA), averaged over consecutive 5 epochs, and matched with the sleep scores. Twenty-second epochs with movement- and arousal related artifacts were visually identified and eliminated. To compute all-night power spectra (0.25-Hz resolution, C3A2 derivation) in nonREM (stages 2, 3 & 4) and REM sleep, all artifact-free 20-s values were averaged. In the recovery nights, data analysis was restricted to the first 8 hours of the 10-hour sleep opportunities.

Waking EEG recordings

During the 40 hours prolonged wakefulness, EEG, EMG, EOC and ECG signals were recorded in 14 sessions at 3-hour intervals in the same way as during the nights, with Rembrandt Datalab® and polygraphic amplifier Artisan®. The first recording was scheduled 15 minutes after lights-on from the baseline night. The study participants were instructed to comfortably relax in a chair, and to place their chin on an individually-adjusted head-rest. A 3-min recording period with eyes closed was followed by a 5-min period with eyes open, while subjects fixated a black dot attached to the wall. At least 1 hour before each waking EEG recording, subjects had to stay in the laboratory (constant temperature: 19-21 °C, light intensity: < 150 lux), and 15 minutes before each recording, they were left by themselves in their bedroom.

The bioelectric signals were conditioned in the same way as in the polysomnographic recordings. Artifacts in all derivations were visually identified and excluded. The power spectra (Fast Fourier Transform, Hanning window) of artifact-free, 50 %-overlapping, 2-s epochs were computed with MATLAB® (The MathWorks Inc, Natick, MA, USA). Mean power spectra between 0-20 Hz (0.5-Hz resolution, C3A2 derivation) of the 5-min periods with eyes open are reported.

Subjective sleepiness and Profile of Mood States

To quantify the evolution of subjective sleepiness, a validated German version of the Stanford Sleepiness Scale (Sturm and Clarenbach 1997) was administered at 3-hour intervals throughout extended wakefulness (first assessment at 08:10 after the baseline night). Subjective sleepiness, vigor, depression and anger were also quantified at 16:45 on days 1 and 2 of prolonged wakefulness with the Profile of Mood States (McNair et al. 1971).

Psychomotor vigilance task (PVT)

All participants completed at 3-hour intervals during extended wakefulness fourteen 10-min sessions of the psychomotor vigilance task (PVT) (Durmer and Dinges 2005). The task was implemented on a PC, using the software e-Prime (Psychology Software Tools Inc., Pittsburgh, PA, USA). When a digital millisecond counter started to scroll in the center of the computer screen, subjects had to press a button with their right forefinger on a response box connected to the PC. In each session, 100 stimuli were presented (random inter-stimulus intervals: 2-10 sec). Subjects received oral instructions and performed one training session on the evening prior to the adaptation night.

Alpha-amylase activity in saliva

Saliva samples (Salivettes®, Sarstedt, Nümbrecht, Germany) were collected at 2-hour intervals throughout prolonged wakefulness, starting at 08:00 after the baseline night. Salivary α-amylase activity (sAA), an indirect marker of sympatho-adrenal activity (van Stegeren et al. 2006) and a recently proposed biomarker of sleep drive (Seugnet et al. 2006), was determined according to previously reported procedures (Nater et al. 2007).

Data analyses and statistics

Cognitive performance, habitual sleep duration, sleep architecture, sleep and waking EEG, subjective sleepiness, mood states, sustained vigilant attention and sAA in baseline and during/after sleep deprivation were analyzed in G/A and G/G genotype subjects. All statistical analyses were performed with SAS® 9.1.3 software (SAS Institute, Cary, NC). Variables that were not normally distributed (absolute EEG power values and response lapses on the PVT) were transformed to approximate a normal distribution. Two- and 3-way, mixed-model analyses of variance (ANOVA) with the between-subjects factor 'gender' (female, male) and the within-subjects factors 'genotype' (G/A, G/G), 'condition' (baseline, recovery/deprivation), 'nonREM sleep episode' (1-4), 'session' (14 assessments during prolonged waking) or 'time' (7 time points of sAA determination) were performed. The significance level was set at $\alpha < 0.05$. If not stated otherwise, only significant effects of factors and interactions are mentioned. Paired and unpaired, 2-tailed *t*-tests to localize differences within and between groups were only performed when the respective main effects and/or interactions of the ANOVA were significant.

Results

The c.22G>A polymorphism of ADA modulates focused attention

Analysis of the d2 attention task in 29 G/A and 191 G/G genotype subjects with right-hand dominance revealed that the G/A genotype processed roughly 30 items less than the G/G genotype

(503 ± 12.6 vs. 534 ± 5.3, $p < 0.04$). This genotype-dependent difference reflects reduced speed in the G/A genotype, and was also present in the participants of the laboratory experiment (see below). By contrast, the number of commission and omission errors did not differ between the groups. Moreover, performance on various tasks reflecting learning, memory, and executive functioning was similar in G/A and G/G genotypes of *ADA* (see supporting information, Table S1)

The c.22G>A polymorphism of ADA does not affect habitual sleep duration

The Munich Chronotype Questionnaire suggested similar sleep length on work days and leisure days in G/A (n = 29) and G/G (n = 191) genotypes ($p_{all} > 0.4$, data not shown). Four-week rest-activity monitoring in the participants of the laboratory study confirmed this notion. Irrespectively of genotype, habitual sleep duration equaled roughly 7.6-7.7 hours when averaged over work and leisure days (see supporting information, Table S3).

The c.22G>A polymorphism of ADA predicts individual differences in slow wave sleep

Both genotype groups showed normal sleep architecture, including short sleep latency and high sleep efficiency in the baseline night (see supporting information, Table S4). Nevertheless, corroborating our previous finding (Rétey et al. 2005), the G/A genotype subjects spent more time in slow wave sleep than the G/G genotype subjects (123.9 ± 7.2 vs. 100.3 ± 6.1 min, $p < 0.001$).

Sleep episode duration, total sleep time, sleep efficiency and slow wave sleep increased in the recovery night after sleep loss when compared to the baseline night. On the contrary, sleep latency, wakefulness after sleep onset, stage 1, stage 2, and REM sleep were reduced. These sleep loss-induced changes in sleep architecture were independent of genotype (see supporting information, Table S4).

The c.G>A polymorphism of ADA predicts higher EEG slow-wave activity in nonREM sleep

To draw conclusions about possible differences in sleep homeostasis between the genotypes, quantitative EEG analyses in sleep and wakefulness are mandatory. Slow-wave (0.75-1.5 Hz) oscillatory activity in nonREM sleep was higher in G/A genotype than in G/G genotype, both in baseline and recovery nights (Fig. 1A). As a physiological marker of sleep homeostasis, slow-wave activity was highest in the first nonREM sleep episode and declined in the course of the night when sleep pressure dissipated (Fig. 2). This time course and the rebound after sleep loss were similar in both genotypes. The data suggest that the c.22G>A polymorphism of *ADA* does not interfere with the dynamics of sleep homeostasis. Nevertheless, the G/A genotype appears to exhibit higher overt sleep pressure than the G/G genotype. Supporting this hypothesis, the *relative* rebound in the first nonREM sleep episode was significantly smaller in A allele carriers than in G/G homozygotes (33.3 ± 7.7 vs. 52.8 ± 6.9 %, $p < 0.05$).

The c.22G>A polymorphism of ADA predicts higher EEG theta/alpha activity in nonREM sleep, REM sleep and wakefulness

The genotype-dependent differences in nonREM sleep were not restricted to the slow wave range, but also included theta and alpha oscillations. Irrespective of normal (baseline night) or elevated (recovery night) sleep pressure, the G/A genotype subjects exhibited higher activity in the entire 6.25-10 Hz band when compared to the G/G genotype subjects (Fig. 1A). Suggesting that this difference reflects altered EEG generating mechanisms rather than a genotype-specific difference in sleep-wake regulation, similar changes were also present in REM sleep (Fig. 1B, 7-12.5 Hz), as well as in wakefulness (Fig. 1C, 8.5-12 Hz). To examine whether the c.22G>A polymorphism of *ADA* affects homeostatic and circadian influences on EEG alpha oscillations in waking (Cajochen et al. 2002), the time course of activity in the 8.5-12 Hz range during extended wakefulness was quantified in G/A and G/G genotypes. Consistent with the conclusion that this genetic variation does not affect the dynamics of sleep-wake regulation, the genotype-dependent difference in alpha activity persisted throughout sleep deprivation and was not modulated by increasing time awake (Fig. 3A).

The c.22G>A polymorphism of ADA predicts higher sleepiness during sleep deprivation

Previous work suggested that increased alpha activity in waking EEG with eyes open may be associated with higher subjective sleepiness, and reduced alertness and sustained attention (Oken et al. 2006). Investigating the evolution of subjective sleepiness during sleep deprivation showed that sleepiness increased in both groups with prolonged time awake and was also modulated by circadian influences. The G/A genotypes, however, were sleepier than the G/G genotypes, particularly after the night without sleep (Fig. 3B). This conclusion was corroborated by the Profile of Mood States (POMS). While sleep loss reduced subjective state in both groups, fatigue was higher and vigor was lower in the G/A genotype than the G/G genotype (Fig. 4). By contrast, the other POMS subscales were not affected by either sleep deprivation or genotype (data not shown).

The c.22G>A polymorphism of ADA predicts reduced sustained attention during sleep deprivation

Performance on the psychomotor vigilance task (PVT) is a sensitive measure of sustained vigilant attention. Reaction times (RT) and number of response lapses (RT > 500 ms) on the PVT were impaired by sleep loss in both *ADA* genotypes. However, consistent with increased EEG alpha activity and elevated subjective sleepiness, G/A genotype subjects performed consistently slower and produced more lapses than G/G genotype subjects throughout prolonged wakefulness (Figs. 3C & 3D). Importantly, the magnitude of the difference between the genotypes was large, comparable to the effects of one night without sleep. The data confirm that tonic alertness is impaired in healthy individuals with genetically reduced adenosine metabolism.

To further support this conclusion, performance on the d2 attention task was separately examined in the participants of the laboratory experiment. Corroborating the finding in the entire study sample, the G/A genotype processed significantly fewer items than the G/G genotype (Fig. 5). This difference reflects reduced speed on the d2 task.

The c.22G>A polymorphism of ADA predicts elevated α-amylase activity in saliva

To investigate whether the c.22G>A polymorphism of *ADA* also affects a recently proposed biomarker of sleep drive (Seugnet et al. 2006), we quantified salivary α-amylase activity (sAA) throughout prolonged wakefulness. We found a pronounced diurnal variation, with highest values in the afternoon and lowest values early at night. Interestingly, sAA in the G/A genotype was significantly higher than in the G/G genotype (Fig. 6). These biochemical data are consistent with our neurophysiological, subjective and behavioral findings, and support the conclusion that the functional G/A polymorphism of *ADA* is associated with elevated sleep pressure.

Discussion

This study demonstrates that healthy adults with genetically-reduced ADA activity (G/A genotype) exhibit higher nonREM sleep pressure than individuals with unimpaired ADA activity (G/G genotype). The carriers of the variant allele have more slow wave sleep, show enhanced brain oscillatory activity within 0.75-1.5 and 6-10 Hz in nonREM sleep (the latter effect is independent of vigilance/sleep state), feel more sleepy and fatigued, are less vigilant (d2 and PVT attention tasks), and present with enhanced α-amylase activity in saliva when compared to G/G homozygotes. These convergent findings demonstrate that in rested and sleep-deprived state, the functional c.22G>A polymorphism of *ADA* not only modulates sleep structure and intensity, but also importantly contributes to inter-individual differences in waking quality, including sleepiness and attention. By contrast, habitual sleep length and the dynamics of the homeostatic response to sleep deprivation are unaffected. Taken together, these data suggest that distinct mechanisms underlie the homeostatic regulation of sleep intensity and sleep duration.

It is widely accepted that EEG slow-wave oscillations are the hallmark of deep nonREM sleep (slow wave sleep) and constitute the primary marker of sleep homeostasis. At the cellular level, slow-wave oscillations consist of depolarized up-states and hyperpolarized down-states in the membrane potential of cortical neurons (Steriade et al. 1993). The up-states last for 0.4-0.8 s and the

down-states last for 0.3-0.7 s (Amzica and Steriade 1998). These membrane fluctuations result in EEG slow oscillations with frequencies of 0.65-1.3 Hz (Bersagliere and Achermann 2010), which perfectly coincide with the frequency range that discriminates the EEG in nonREM sleep between G/A and G/G genotypes of *ADA*. Intra-cellular recordings in non-anaesthetized cats suggest that the long-lasting hyperpolarized down-states represent periods of disfacilitation, a form of inhibition due to reduced activating input from ascending cholinergic and monoaminergic pathways (Steriade et al. 2001; Timofeev et al. 2001). Extracellular adenosine could contribute to cortical disfacilitation by inhibiting basal forebrain and mesopontine cholinergic neurons (Rainnie et al., 1994). Thus, the functional c.22G>A polymorphism of *ADA* may enhance endogenous adenosine levels and amplify cortical disfacilitation in deep nonREM sleep.

Genetic studies in mice and pharmacological experiments in rats and humans also support a role for adenosine in modulating low-frequency slow-wave oscillations in nonREM sleep. Recent insights suggest that integrated brain circuits consisting of neurons and astrocytes regulate extracellular adenosine and adenosine-mediated modulation of neural transmission (Haydon and Carmignoto 2006; Halassa and Haydon 2010). Astrocytes can be activated by neurotransmitters from adjacent synapses and release chemicals by a process called gliotransmission. The primary molecules released by gliotransmission are glutamate, ATP and D-serine (Oliet and Mothet 2006). The ATP is rapidly hydrolyzed to adenosine and modulates synaptic activity by acting on adenosine receptors. Astrocytic modulation of cortical synapses is relevant for sleep-related EEG rhythms *in vivo* (Halassa and Haydon 2010) More specifically, absence of gliotransmission by dominant-negative inhibition of SNARE-dependent membrane fusion (dnSNARE) exclusively in astrocytes decreases the slow oscillation in somatosensory cortex (Fellin et al. 2009). Intriguingly, the dnSNARE mice show reduced slow-wave activity in nonREM sleep in baseline, as well as in recovery sleep after sleep deprivation (Halassa et al. 2009). Reminiscent of the genotype-dependent difference between G/A and G/G allele carriers of *ADA*, the difference to wild-type mice is most pronounced in the 0.5-1.5 Hz band in nonREM sleep.

Last but not least, the adenosine A_1 and A_{2A} receptor antagonist, caffeine, is well-known to reduce primarily EEG low-delta frequencies in nonREM sleep in rested and sleep deprived states (rats: 0.75-1.5 Hz; humans: 0.75-2.0 Hz) (Schwierin et al. 1996; Landolt et al. 1995; Landolt et al. 2004). Genetic studies suggest that A_{2A} receptors play a primary role for the effects of caffeine on sleep. Mice with A_{2A} receptor loss-of-function have reduced sleep and blunted response to sleep deprivation, as well as to the wake-promoting effects of caffeine (Urade et al. 2003; Huang et al. 2005). Consistent with this notion, the human c.1976T>C polymorphism (rs5751876) of the A_{2A} receptor gene (*ADORA2A*) modulates individual sensitivity to caffeine on sleep (Rétey et al. 2007). This polymorphism has also a striking effect on EEG theta/low-alpha (~ 6.5-9.5 Hz) oscillations in nonREM sleep, REM sleep and wakefulness (Rétey et al. 2005). The partial overlap of this frequency range with the findings in the present study suggests that the repercussions of genetically-altered ADA activity may, at least in part, be mediated by adenosine A_{2A} receptors.

The consequences of genetic abolition of gliotransmission on EEG activity in REM sleep and wakefulness in mice are unknown. Remarkably, however, performance on a novel object recognition task appears virtually unaffected after prolonged waking in dnSNARE mice when compared to wild-type animals (Halassa et al. 2009). This phenotype may be reminiscent of the difference in performance on d2 and PVT attention tasks between human G/A allele carriers and G/G homozygotes of *ADA*. Although the comparison between exploration of novel objects in rodents and attention performance in humans needs to be made with caution, our findings could indicate that the c.22G>A polymorphism increases overt homeostatic sleep pressure by interfering with the astrocyte-dependent regulation of extracellular adenosine. Indeed, ADA may be more abundantly expressed in astrocytes than in neurons (Fredholm et al. 2005).

Slow-wave oscillations in nonREM sleep are highest in the beginning of the night and most pronounced in those brain areas that were disproportionally active during preceding wakefulness (Kattler et al. 1994; Ferrara et al. 2002; Huber et al. 2004). Moreover, experimental induction of slow waves may potentiate the beneficial effects of sleep (Marshall et al. 2006; Walsh et al. 2006; Walsh et al. 2010), whereas their suppression may impair proper functions of brain and body (Aeschbach et al.

2008; Tasali et al. 2008; Landsness et al. 2009; Van Der Werf et al. 2009). Thus, slow-wave oscillations in nonREM sleep are thought to reflect a restorative function of sleep. Based upon this reasoning, the physiologic enhancement of sleep pressure and slow-wave oscillations may be a promising strategy to potentiate the positive effects of sleep. Because adenosine and adenosine receptors play a well-established role in sleep homeostasis, this neuromodulatory system could provide a rational target to intensify sleep, for example in patients with insomnia, shallow sleep and disturbed vigilance. Apart from possible unrelated unwanted reactions (Landolt 2008), the present genetic study shows that such an approach would likely impair the quality of wakefulness. More specifically, waking functions (e.g., sustained attention) could be reduced to an extent that is similar to the effect of one night without sleep. Indeed, with respect to another gene variant possibly involved in sleep-wake regulation, $PER3^{5/5}$ genotype subjects of the circadian clock gene *PERIOD-3* not only exhibit more slow wave sleep and EEG low-frequency activity, but are also more impaired by sleep deprivation than $PER3^{4/4}$ homozygotes (Viola et al. 2007). Our findings demonstrate that a similar sleep phenotype can impair waking performance even in well-rested individuals.

In conclusion, functional polymorphic variation of *ADA* in healthy adults distinctly affects nonREM sleep intensity, EEG theta/alpha frequencies in sleep and wakefulness, attention, subjective sleepiness, and α-amylase activity in saliva. These differences do not mirror differences in habitual sleep duration and are robust against the effects of sleep deprivation. Thus, they do not reflect a genotype-dependent alteration in the dynamics of sleep homeostasis. This observation is consistent with recent findings in monozygotic and dizygotic twins, showing that the pronounced genetic influences on the sleep EEG are independent of elevated sleep pressure after sleep loss (De Gennaro et al. 2008). Moreover, as shown in rats (Mackiewicz et al. 2003), Ada enzymatic activity is not affected by sleep deprivation. The data rather suggest an elevated level in overt, homeostatically-regulated nonREM sleep propensity in the G/A genotype compared to G/G homozygotes, which may be due to elevated adenosinergic tone at the synapse because of genetically-reduced ADA activity. Whether this difference directly underlies the observed phenotypes in sleep and wakefulness, or

whether it modulates other molecular systems contributing to the homeostatic regulation of sleep propensity, remains to be elucidated.

Acknowledgments

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Caption to figures

Figure 1. The functional c.22G>A polymorphism of *ADA* modulates EEG activity in nonREM sleep, REM sleep and wakefulness. EEG power density (C3A2 derivation) between 0-20 Hz in the G/A genotype (n = 11) was expressed as a percentage of the corresponding values in the G/G genotype (n = 11; horizontal dashed line at 100%). Data in nonREM (**A**) (stages 2, 3, 4) and REM sleep (**B**) represent all-night values in baseline (white symbols) and recovery nights (black symbols). In the waking EEG (**C**), averaged power over five 5-min recordings at 8 am, 11 am, 2 pm, 5 pm and 8 pm on day 1 (baseline, white squares) and day 2 (deprivation, black squares) during prolonged wakefulness are represented. Geometric means are plotted for each 0.25 Hz bin in nonREM and REM sleep, and for each 0.5 Hz bin in wakefulness. Black triangles denote a significant effect of *'genotype'* ($F_{1,30} \geq 4.2$, $p < 0.05$) of a 2-way, mixed-model ANOVA with the within-subject factors *'genotype'* (G/A, G/G) and *'condition'* (baseline, recovery/deprivation).

Figure 2. The functional c.22G>A polymorphism of *ADA* predicts higher low-delta activity (C3A2 derivation, power within 0.75-1.5 Hz) in nonREM sleep (stages 2-4). By contrast, it does not affect the time course and the sleep loss-induced rebound of EEG slow wave oscillations after sleep deprivation. Mean delta activity in G/A (left panel) and G/G genotypes (right panel) in nonREM sleep episodes 1-4 in baseline (grey bars) and recovery nights (black bars) is plotted. Error bars represent 1 SEM (n = 11). Three-way, mixed-model ANOVA with the within-subject factors *'genotype'* (G/A, G/G), *'condition'* (baseline, recovery) and *'nonREM sleep episode'* (1-4) confirmed the significant effect of *'genotype'* (*'genotype'*: $F_{1,44.2} = 8.8$, $p < 0.005$).

** $p < 0.01$ (recovery *vs.* baseline; paired, 2-tailed *t*-test)

*** $p < 0.001$ (recovery *vs.* baseline; paired, 2-tailed *t*-test)

Figure 3. The functional c.22G>A polymorphism of ADA predicts higher EEG alpha activity, elevated subjective sleepiness and impaired sustained attention during prolonged wakefulness. Starting 15 minutes after wakening from the baseline night, 14 test sessions at 3-hour intervals consisting of 5-min waking EEG recording, subjective sleepiness rating and testing of sustained attention were completed in each individual. Ticks on the x-axis are rounded to the nearest hour.

Black circles: G/A genotype (n = 11). Grey circles: G/G genotype (n = 11). Data were analyzed with 2-way, mixed-model ANOVA with the within-subject factors *'genotype'* (G/A, G/G) and *'session'* (14 assessments during prolonged waking). (**A**) Throughout prolonged wakefulness, EEG activity in the 8.5-12 Hz range was consistently higher in G/A genotype than in G/G allele carriers (*'genotype'*: $F_{1,30}$=10.9, $p < 0.003$; *'session'*: $F_{13,239}$=2.3, $p < 0.007$; *'genotype'* x *'session'* interaction: $F_{13,159}$=0.2, $p > 0.9$). (**B**) The evolution of subjective sleepiness during sleep deprivation was quantified with the Stanford Sleepiness Scale. ANOVA revealed significantly higher sleepiness in the G/A genotype than in the G/G genotype (*'genotype'*: $F_{1,78}$= 6.3, $p < 0.02$; *'session'*: $F_{13,155}$= 42.1, $p < 0.001$; *'genotype'* x *'session'* interaction: $F_{13,162}$= 1.7, $p < 0.08$). The difference becomes evident after the night without sleep. (**C**) & (**D**) Sustained attention during prolonged wakefulness was quantified with the PVT. The time courses of speed (1/median reaction time [RT]) and response lapses (RT > 500 ms, transformed by $\sqrt{x} + \sqrt{(1+x)}$) are illustrated. All RT < 100 ms ("errors of commission") were excluded from analyses. The G/A genotype performed significantly worse that the G/G genotype throughout prolonged waking (speed: *'genotype'*: $F_{1,25}$= 15.4, $p < 0.001$; *'session'*: $F_{13,239}$= 38.6, $p < 0.001$; *'genotype'* x *'session'* interaction: $F_{13,146}$= 0.3, $p > 0.9$; lapses: *'genotype'*: $F_{1,66}$= 24.5, $p < 0.001$; *'session'*: $F_{13,194}$= 19.5, $p < 0.001$; *'genotype'* x *'session'* interaction: $F_{13,144}$= 1.1, $p > 0.3$).

Figure 4. Elevated fatigue and reduced vigor in G/A genotype (n = 11) compared to G/G genotype (n = 11) of *ADA*. The Profile of Mood States (POMS) was administered at 16:45 on days 1 (baseline, grey bars) and 2 (deprivation, black bars) of extended wakefulness. Data represent means + SEM. They were analyzed with 2-way, mixed-model ANOVA with the within-subject factors *'genotype'* (G/A, G/G) and *'condition'* (baseline, deprivation). In rested and sleep-deprived state, fatigue was higher and vigor was lower in the G/A genotype (fatigue: *'genotype'*: $F_{1,30}$= 4.2, $p < 0.05$ [*]; *'condition'*: $F_{1,30}$= 28.8, $p < 0.001$; *'genotype'* x *'condition'* interaction: $F_{1,30}$= 0.0, $p > 0.8$; vigor: *'genotype'*: $F_{1,30}$= 9.2, $p < 0.005$ [**]; *'condition'*: $F_{1,30}$= 16.1, $p < 0.001$; *'genotype'* x *'session'* interaction: $F_{1,30}$= 0.0, $p > 0.8$).

Figure 5. Reduced speed on d2 attention task in the G/A genotype compared to the G/G genotype of *ADA*. The upper and lower lines of the "box and whisker plots" represent the 75th and 25th percentiles

of the study sample, whereas the horizontal lines in the middle of the boxes indicate the sample medians (50^{th} percentiles). Mean values of processed items (dashed lines) differed significantly between 11 G/A (white box) and 9 G/G (grey box) genotype subjects (484 ± 19.1 vs. 561 ± 22.6, $p < 0.02$; paired, 2-tailed *t*-test). By contrast, the number of errors did not differ between the groups.

Figure 6. Elevated salivary α-amylase activity (sAA) in G/A genotype compared to G/G genotype of *ADA*. Saliva samples were collected at 2-hour intervals, starting at 8 am on day 1 of prolonged wakefulness. The sAA (U/ml) was averaged per 6-hour intervals. Error bars represent SEM (n = 11). The data were analyzed with 2-way, mixed-model ANOVA with the within-subject factors *'genotype'* (G/A, G/G) and *'time'* (7 time points during prolonged waking). ANOVA revealed higher sAA in the G/A genotype (*'genotype'*: $F_{1,21}=5.2$, $p < 0.04$; *'time'*: $F_{6,97}= 12.6$, $p < 0.001$; 'genotype' x 'time' interaction: $F_{6,71}= 0.7$, $p > 0.6$).

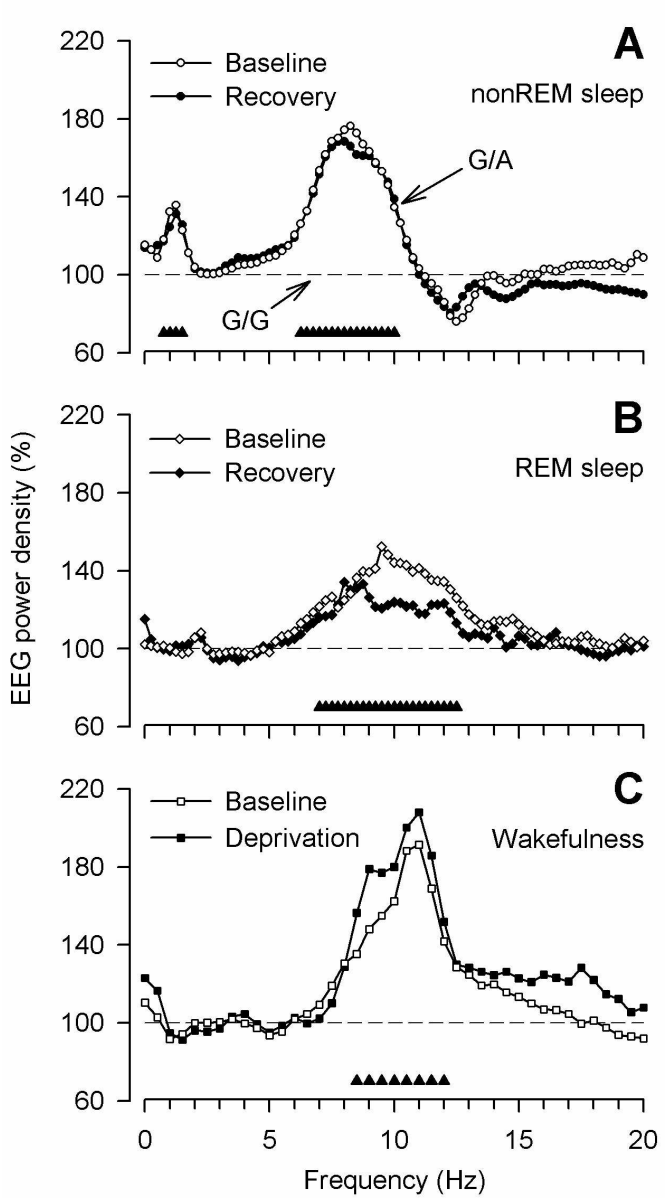

Figure 1
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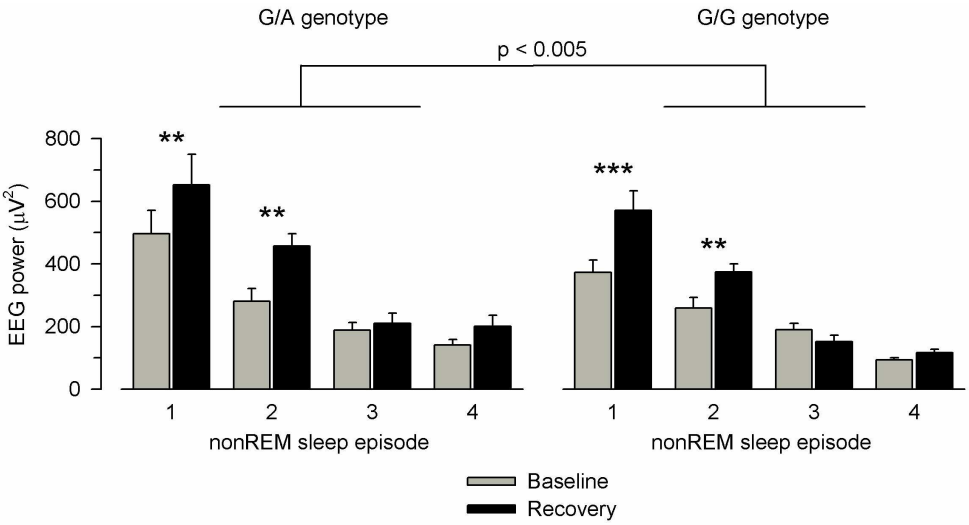

Figure 2, revised
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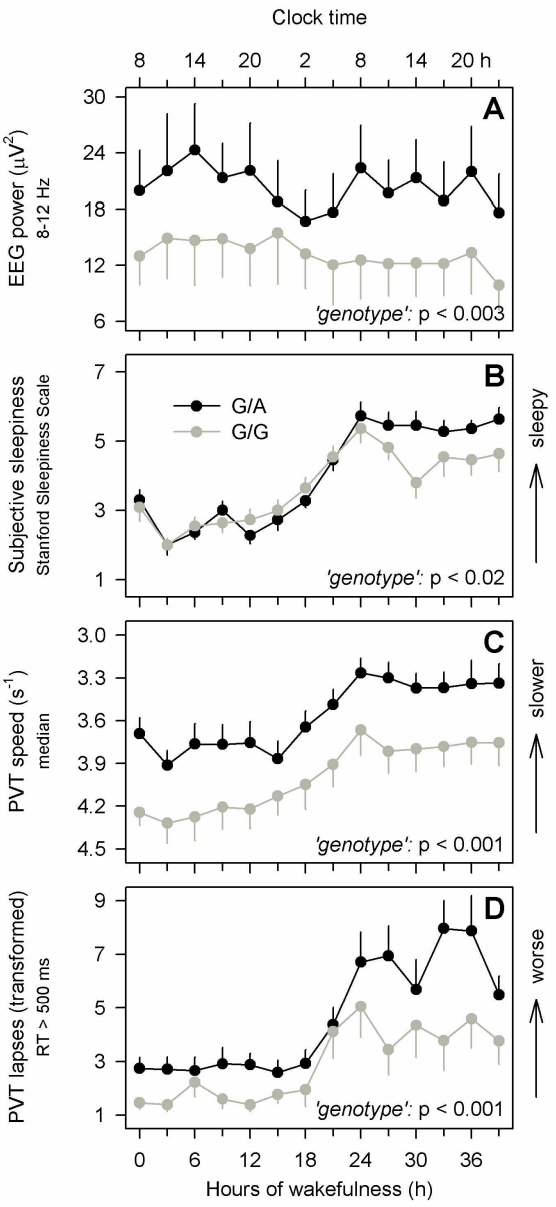

Figure 3
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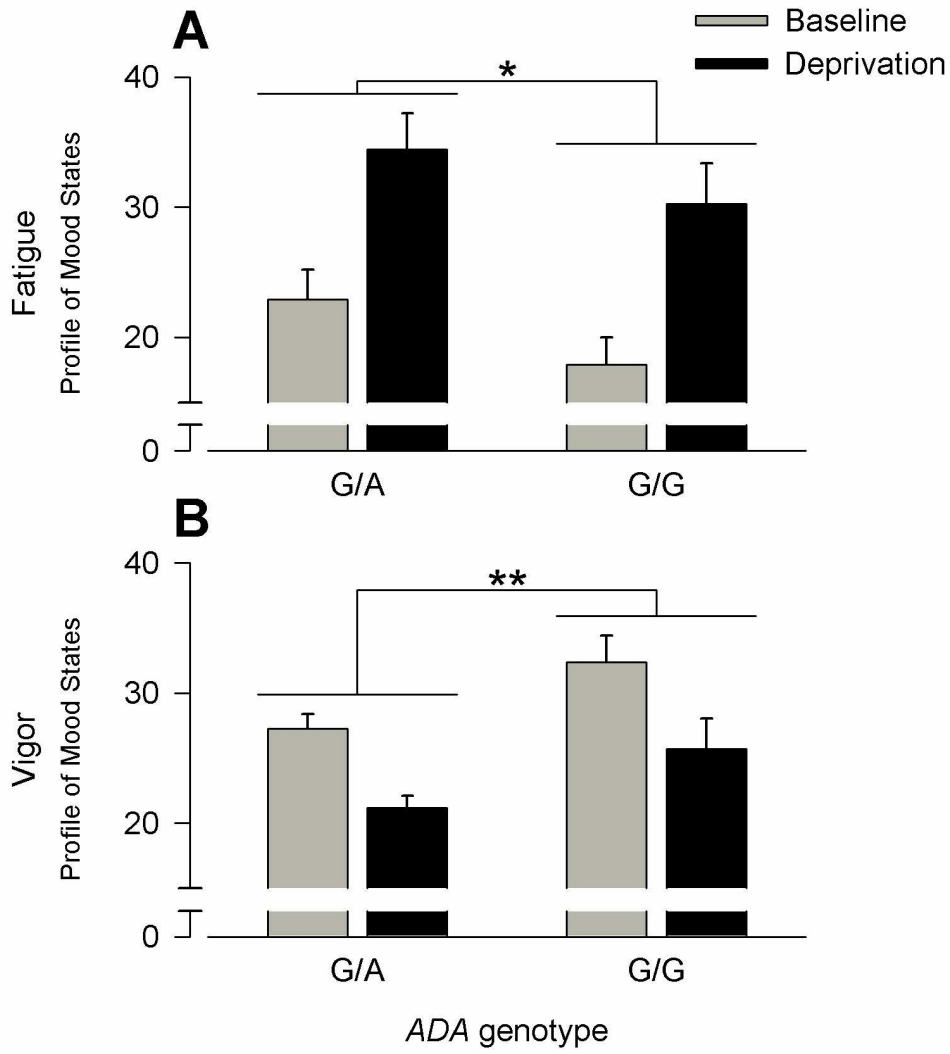

Figure 4
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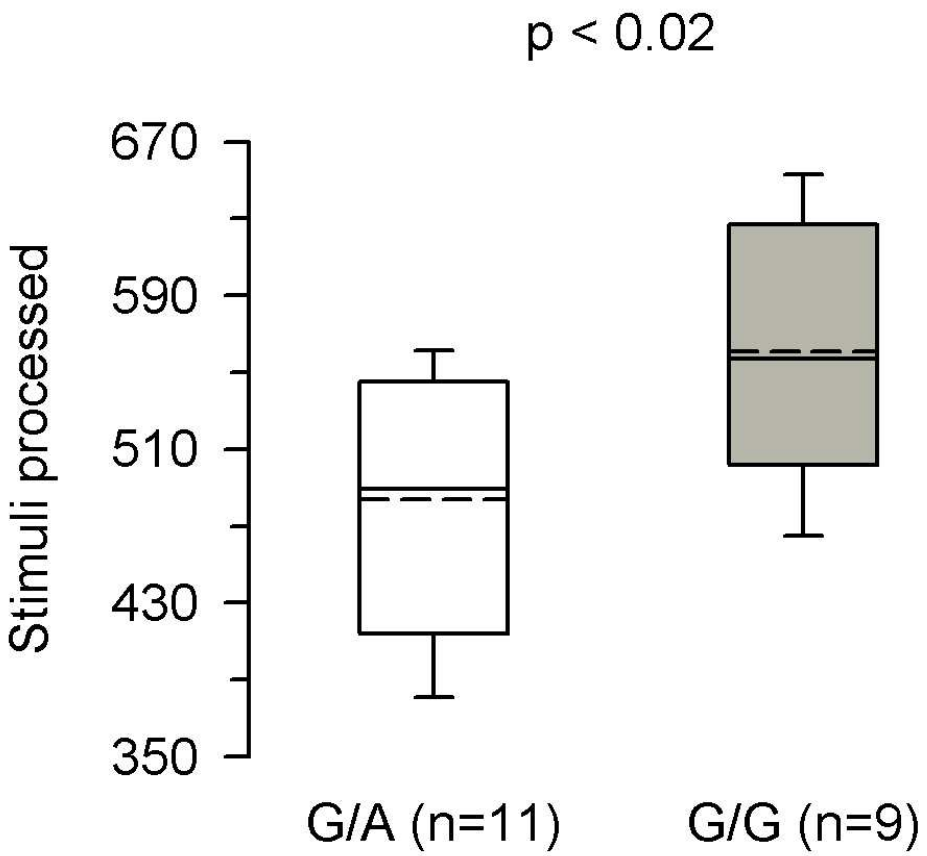

Figure 5
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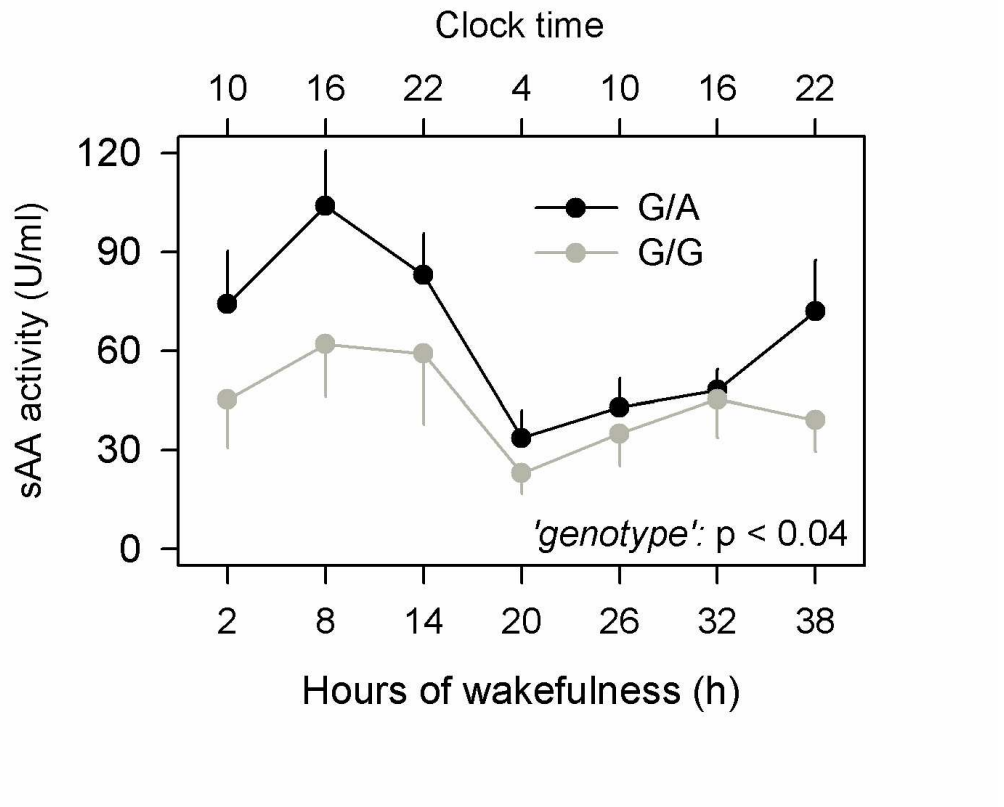

Figure 6, revised
233x189mm (150 x 150 DPI)

FUNCTIONAL *ADA* POLYMORPHISM INCREASES SLEEP DEPTH AND REDUCES VIGILANT ATTENTION IN HUMANS

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Supplementary material

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Table S1. Cognitive assessment of study participants.

	G/A genotype	G/G genotype	*'genotype'*	*'gender'*	*'genotype*gender'*
Attention					
d2 Task:			$F_{1, 216}$ (p)	$F_{1, 216}$ (p)	$F_{1, 216}$ (p)
Number of processed items	503.2 ± 12.4	533.7 ± 5.3	4.6 (0.03)	1.0 (0.31)	0.3 (0.60)
Sum of omission and commission errors	21.4 ± 3.3	24.0 ± 1.4	0.6 (0.45)	0.7 (0.40)	2.0 (0.16)
Total number of items processed minus errors	481.8 ± 12.5	509.6 ± 5.5	3.6 (0.06)	1.4 (0.23)	0.0 (0.87)
Fluctuation rate	11.4 ± 0.7	11.5 ± 0.3	0.0 (0.93)	0.1 (0.78)	0.2 (0.66)
Learning efficiency and memory decline					
Rey Auditory Verbal Learning Test (RAVLT)			$F_{1, 216}$ (p)	$F_{1, 216}$ (p)	$F_{1, 216}$ (p)
Total number of total words recalled across five trials	56.7 ± 1.5	55.5 ± 0.6	0.6 (0.45)	3.6 (0.06)	0.0 (0.93)
Immediate recall	12.6 ± 0.3	12.5 ± 0.1	0.1 (0.78)	6.8 (0.01)	0.2 (0.68)
Delayed recall	12.7 ± 0.4	12.3 ± 0.2	1.1 (0.29)	12.3 (0.001)	0.9 (0.34)
Rey Visual Design Learning Test (RVDLT)					
Total number of total figures recalled across five trials	55.7 ± 2.0	55.5 ± 0.6	0.2 (0.64)	1.6 (0.20)	1.2 (0.27)
Immediate recall	13.1 ± 0.5	13.1 ± 0.2	0.0 (0.86)	1.9 (0.17)	1.5 (0.22)
Delayed recall	13.1 ± 0.5	13.2 ± 0.2	0.0 (0.93)	1.7 (0.19)	1.3 (0.26)
Working memory					
Digit span test			$F_{1, 215}$ (p)	$F_{1, 215}$ (p)	$F_{1, 215}$ (p)
Number of digits recalled during forward run	6.6 ± 0.2	6.8 ± 0.1	0.8 (0.37)	0.7 (0.40)	0.2 (0.70)
Number of digits recalled during backward run	5.6 ± 0.3	5.6 ± 0.1	0.0 (0.91)	1.4 (0.24)	7.6 (0.01)

Table S1. Cognitive assessment of study participants (continued).

	G/A genotype	G/G genotype	*'genotype'*	*'gender'*	*'genotype*gender'*
Executive functioning					
Stroop color-word test			$F_{1, 216}$ (p)	$F_{1, 216}$ (p)	$F_{1, 216}$ (p)
Interference score in milliseconds (s)	6.4 ± 0.6	7.0 ± 0.3	0.5 (0.49)	1.6 (0.21)	0.1 (0.76)
Random Number Generation Test			$F_{1, 216}$ (p)	$F_{1, 216}$ (p)	$F_{1, 216}$ (p)
Redundancy	0.9 ± 0.1	0.8 ± 0.0	0.5 (0.50)	0.4 (0.54)	0.1 (0.74)
Adjacency	35.0 ± 1.2	38.1 ± 0.7	3.1 (0.08)	0.0 (0.99)	2.5 (0.11)
Go/ no-go Task			$F_{1, 209}$ (p)	$F_{1, 209}$ (p)	$F_{1, 209}$ (p)
Reaction time in milliseconds (ms)	429.0 ± 6.2	418.2 ± 3.3	1.5 (0.23)	0.6 (0.45)	1.7 (0.19)
Nonverbal fluency test (5 point test)			$F_{1, 216}$ (p)	$F_{1, 216}$ (p)	$F_{1, 216}$ (p)
Sum of admissible figures designed during 3 minutes	44.7 ± 1.9	45.2 ± 0.7	0.1 (0.83)	0.1 (0.72)	0.1 (0.70)
Verbal fluency test (s word test)			$F_{1, 216}$ (p)	$F_{1, 216}$ (p)	$F_{1, 216}$ (p)
Sum of admissible words produced during 3 minutes	34.0 ± 1.7	35.6 ± 0.7	0.7 (0.42)	6.5 (0.01)	3.6 (0.06)

Table S2. Demographic characteristics of participants in sleep study.

	22G>A genotype		*p*-value
	G/A	G/G	
Sex ratio (females / males)	5/6	5/6	n/a
Age (years)	24.3 ± 1.2	24.5 ± 1.0	0.7
Years of education	12.1 ± 0.5	12.7 ± 0.5	0.4
Alcohol consumption (drinks/week)	4.0 ± 0.9	2.6 ± 0.6	0.2
Caffeine consumption (mg/day)	149.6 ± 33.6	181.4 ± 38.4	0.6
Body mass index (kg/m^2)	21.8 ± 0.6	21.9 ± 0.4	0.9
Trait Anxiety	33.0 ± 1.9	35.5 ± 2.4	0.5
Chronotype	2.6 ± 0.2	3.0 ± 0.2	0.1
Daytime sleepiness	6.5 ± 1.0	5.0 ± 0.8	0.3

Values represent means ± SEM (n = 11 per group).

Values of caffeine consumption were based on the following average caffeine content per serving: Coffee: 100 mg; Ceylon or green tea: 30 mg; Cola drink: 40 mg (2 dl); Energy drink: 80 mg (2 dl); Chocolate: 50 mg (100 g). Trait anxiety, diurnal preference ("chronotype") and daytime sleepiness were assessed with the Trait-State Anxiety Inventory (Spielberger et al. 1970), the Horne-Östberg Morningness-Eveningness Questionnaire (Horne and Östberg 1976) and the Epworth Sleepiness Scale (Johns 1991). The chronotypes were classified based upon the absolute Horne-Östberg score (in parentheses): 1 = definite evening type (16-30), 2 = moderate evening type (31-41), 3 = neutral type (42-58), 4 = moderate morning type (59-69), 5 = definite morning type (70-86).

P-values refer to paired, 2-tailed *t*-tests.

Table S3. The 22G>A polymorphism of *ADA* does not affect habitual sleep duration.

Sleep duration	G/A genotype		G/G genotype	
	MCTQ	Actigraphy	MCTQ	Actigraphy
Work days	7.2 ± 0.4	7.3 ± 0.2	7.3 ± 0.3	7.5 ± 0.2
Leisure days	8.6 ± 0.4	8.2 ± 0.3	8.2 ± 0.3	8.2 ± 0.4
Average over work and leisure days	7.6 ± 0.3	7.6 ± 0.2	7.6 ± 0.3	7.7 ± 0.2

Mean values ± SEM (hours) in 11 G/A and 11 G/G allele carriers.

Average values over work and leisure days were calculated by weighing 5 work and 2 leisure days. MCTQ: Munich ChronoType Questionnaire (Roenneberg et al. 2003). To objectively estimate habitual sleep duration, volunteers continuously wore a rest-activity monitor (actigraphy) and kept a sleep-wake diary during 4 weeks at home. The monitor data were missing in 2 G/G genotype subjects. Sleep duration refers to the difference between start and end times of the estimated nocturnal rest episode. In both genotypes, MCTQ and actigraphy revealed longer sleep on leisure days than on work days ($p_{all} < 0.006$, paired 2-tailed t-tests).

Table S4. Visually scored sleep variables in baseline and recovery nights.

Variable	G/A genotype		G/G genotype		*'genotype'*	*'condition'*	*'genotype' x 'condition'*
	Baseline	Recovery	Baseline	Recovery	$F_{1,30}$ (p <)	$F_{1,30}$ (p <)	$F_{1,30}$ (p <)
Sleep episode	466.1 ± 4.4	476.2 ± 0.8	463.0 ± 4.3	476.9 ± 0.5	0.2 (0.70)	14.7 (0.001)	0.4 (0.55)
Total sleep time	453.6 ± 5.7	466.7 ± 1.3	447.7 ± 4.4	465.9 ± 1.2	0.8 (0.37)	18.8 (0.001)	0.5 (0.49)
Sleep efficiency	94.6 ± 1.2	97.2 ± 0.3	93.4 ± 0.9	97.1 ± 0.2	0.9 (0.36)	18.0 (0.001)	0.5 (0.48)
Sleep latency	13.3 ± 4.4	3.8 ± 0.8	16.5 ± 4.3	3.2 ± 0.5	0.2 (0.69)	13.6 (0.001)	0.4 (0.55)
REM latency	76.1 ± 8.9	91.2 ± 13.1	73.9 ± 7.3	73.2 ± 8.8	1.1 (0.31)	0.5 (0.47)	0.7 (0.43)
WASO	3.9 ± 1.6	0.5 ± 0.2	4.2 ± 0.6	1.0 ± 0.3	0.1 (0.72)	8.6 (0.01)	0.01 (0.94)
Stage 1	32.2 ± 3.9	20.1 ± 5.3	31.7 ± 3.9	16.2 ± 3.2	0.4 (0.52)	17.6 (0.001)	0.3 (0.61)
Stage 2	196.6 ± 7.9	162.7 ± 4.5**	212.1 ± 4.8	207.1 ± 6.2	26.7 (0.001)	11.3 (0.003)	6.2 (0.02)
Slow wave sleep	123.9 ± 7.2**	191.2 ± 8.2**	100.3 ± 6.1	153.0 ± 4.2	51.6 (0.001)	194.7 (0.001)	2.9 (0.11)
REM sleep	100.9 ± 5.4	92.8 ± 3.8	103.6 ± 4.2	89.7 ± 7.0	0 (0.98)	4.5 (0.05)	0.3 (0.59)
Movement time	8.7 ± 0.8	9.0 ± 0.9	11.1 ± 1.7	9.9 ± 1.1	3.0 (0.10)	0.2 (0.68)	0.7 (0.43)

Mean values ± SEM in 11 G/A and 11 G/G allele carriers in minutes (except sleep efficiency [%]) for the first 480 minutes from lights-off. Baseline: baseline night. Recovery: recovery night after 40 hours prolonged wakefulness. Sleep episode: time after sleep onset until final awakening. Sleep efficiency: total sleep time per 480 min. Sleep latency: time from lights-off to first occurrence of stage 2 sleep. REM latency: time from sleep onset to first occurrence of REM sleep. WASO: waking after sleep onset. Slow wave sleep: combined stages 3 & 4.

F- and p-values: 2-way mixed-model ANOVA with within-subjects factors *'genotype'* (G/A, G/G) and *'condition'* (baseline, recovery).

** $p < 0.001$ (G/A *vs.* G/G genotype; paired, 2-tailed t-test)

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